

# Relation of smoking to immunoreactive endothelin in the bronchiolar epithelial cells

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## Abstract

**Background** – Endothelin is a potent bronchoconstrictor which appears to be important in asthma. To ascertain whether cigarette smoking is associated with any alteration in the proportion of bronchiolar epithelial cells which express endothelin immunoreactivity, the airways in the lungs of non-smokers and smokers were analysed. Since an increase in immunoreactivity has been found in the bronchial epithelial cells of asthmatic subjects, cigarette smokers with and without evidence of airway hyperresponsiveness were also selected.

**Methods** – A point counting method which examined the proportion of endothelin immunoreactive epithelial cells in membranous and respiratory bronchioles was used.

**Results** – Neither smoking itself nor evidence of airway hyperresponsiveness altered the percentage of endothelin immunoreactive epithelial cells in the membranous and respiratory bronchioles.

**Conclusions** – Cigarette smoke does not induce endothelin production in bronchiolar epithelial cells, and the airway hyperresponsiveness seen in some patients with lung disease induced by cigarette smoking is not related to exaggerated endothelin production in epithelial cells.

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Endothelins are a group of peptides which produce considerable bronchoconstriction.<sup>1</sup> Bronchial epithelial cells have been found to produce the peptide in culture,<sup>2</sup> and morphological studies have shown immunoreactive peptide in the bronchial and bronchiolar cells of human airways.<sup>3</sup> Furthermore, bronchial smooth muscle cells have specific binding sites for endothelin,<sup>4</sup> and endothelin is known to act as a potent mitogen for smooth muscle cells.<sup>5</sup> Airways responsiveness also appears to be important in the alteration of pulmonary function seen in cigarette smokers. As a result of the known similarity in the airways disease of asthmatic subjects and cigarette smokers<sup>6-8</sup> we speculated that cigarette smoking may produce an increased expression of endothelin in the bronchiolar epithelial cells. A morphometric analysis was therefore performed to examine the proportion of immunoreactive epithelial cells in the respiratory and membranous bronchioles in (1) groups of non-smokers and smokers with

no knowledge of their bronchial responsiveness, and (2) groups of non-smokers and smokers in whom bronchial responsiveness had been measured before pulmonary resection by methacholine challenge.

## Methods

The patients were part of a larger structure function study in our institution. All had undergone lobectomy or pneumonectomy for a mass lesion, usually carcinoma. For the initial study five lifetime non-smokers were selected and matched for age with an equal number of current smokers who had a wide range of smoking histories. Smoking history was expressed as pack years.

For the second study subjects were selected from a subgroup of patients who had performed a methacholine challenge before surgery. Matched with the general smokers for total smoking history, a group of five smokers were selected who had responded to low concentrations of methacholine (range 0.23–1.48, geometric mean 0.53, arithmetic mean 0.74 mg/ml) with a drop of 20% in their FEV<sub>1</sub>, and a second group of five patients who did not exhibit such a response (methacholine PC<sub>20</sub> range 14.9–>16 mg/ml).

All lungs were treated in a similar manner. They were fixed in inflation with buffered formalin and random sections were taken from the medial and lateral slices and processed in paraffin. Sections 4 µm thick were mounted on slides and immunostained for endothelin reactivity by the avidin-biotin-peroxidase (ABP) technique. In brief, sections were first deparaffinised and endogenous peroxidase was blocked by incubation with hydrogen peroxide. After washing with phosphate buffered saline (PBS) the sections were incubated overnight at room temperature with anti-endothelin antibody (Serotec) in a dilution of 1:4000. Following a PBS wash the sections were incubated for one hour at room temperature with biotinylated antimouse IgG (Vectastain), washed with PBS, the ABP complex (Vectastain Kit, Vector, USA) applied, and peroxidase activity revealed using 3-amino-9-ethylcarbazole chromogen. Sections were then counterstained with haematoxylin, dehydrated, cleared, and coverslipped. Negative controls were constructed by omission of the primary antibody.

The sections were examined under low power and the position of each respiratory and membranous bronchiole was identified. Using a random number generator five respiratory and membranous bronchioles were selected for analysis. With a 100 × oil immersion lens and a 42

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Mean (SD) and range of patient data and proportion of immunoreactive bronchiolar epithelial cells in respiratory bronchioles (RB) and membranous bronchioles (MB)

	Non-smokers (n = 5)	Smokers (general) (n = 5)	Reactive smokers (n = 5)	Non-reactive smokers (n = 5)
Age (years)	63 (6)	62 (8)	69 (8)	51 (10)
Pack years	0	44 (18) (26–70)	44 (21) (23–74)	44 (18) (21–66)
FEV <sub>1</sub> (% pred)	108 (17)	66 (17)	63 (11)	82 (21)
RB (% positive)	65 (21) (13–94)	72 (8) (58–88)	41 (20) (7–82)	42 (19) (8–69)
MB (% positive)	72 (19) (30–100)	72 (11) (42–89)	52 (29) (13–94)	49 (26) (2–85)

FEV<sub>1</sub> = forced expiratory volume in one second.

point grid five random sites along the length of the bronchiole were chosen and the grid points falling on the epithelial cells were classified as positive if the cell contained recognisable granules of peroxidase. By this method a mean of 32 points per airway (range 17–61) for the membranous bronchioles and a mean of 22 points per airway (range 12–37) in the respiratory bronchioles were examined. The data for the five sites of each airway were summed, and the proportion of immunoreactive cells for that airway calculated. The numbers of sites and airways were calculated to give a standard error 5% of the mean. Such methodology represents standard morphometric technique<sup>9</sup> and can be used as an alternative to the subjective techniques used in other studies.<sup>10,11</sup>

Data were analysed by the SYSTAT statistical system.<sup>12</sup> The Kolmogorov-Smirnov test was used to test whether the distribution of the proportion of endothelin immunoreactive cells in the airways was different between the groups.

## Results

The patient data are summarised in the table. The mean age of the patients in each group was similar, and the groups of patients who smoked had similar cumulative smoking histories. The table also shows the proportion of epithelial cells in the membranous and respiratory bronchioles which expressed endothelin immunoreactivity. No group showed any difference between the distribution of the proportion of reactive cells in respiratory compared with membranous bronchioles. The distribution of immunoreactive cells in the smokers with reactive airways was similar to that found in the group of smokers with non-reactive airways.

## Discussion

Endothelin appears to have a major role in maintenance of bronchial tone as well as in the physiological and pathological changes of asthma. This hypothesis was supported by the studies of Springall and colleagues<sup>11</sup> who found that bronchial biopsy specimens from asthmatic subjects had a significantly increased expression of endothelin immunoreactivity. Nomura *et al*<sup>13</sup> analysed the bronchoalveolar lavage fluid of a patient during an asthmatic attack and also found increased amounts of endothelin compared with the values after recovery.

The recently published multicentre clinical

trial (Lung Health Study) showed that current smokers with functional evidence of early chronic obstructive pulmonary disease (COPD) had non-specific airways hyperresponsiveness which was related to the baseline values for lung function.<sup>14</sup> Other longitudinal studies have indicated that airway hyperreactivity<sup>15</sup> or lack of response to bronchodilators<sup>15,16</sup> are important predictors of an increased rate of decline in FEV<sub>1</sub>, independent of the effects of smoking, and that these patients also have a greater mortality than patients without such responsiveness. Similarly, a recent study of young cigarette smokers<sup>17</sup> has shown that the presence of wheezing – probably indicative of lung hyperresponsiveness – is predictive of a progressive loss of ventilatory function. Because of the similarities between asthma and COPD we hypothesised that those patients whose epithelium had been stimulated to produce and secrete endothelin would manifest an increased degree of bronchoconstriction.

Although we were only able to include groups of five patients, and it is possible that we could miss a subtle change, our data do not support this. The proportion of immunoreactive epithelial cells was similar in non-smokers and current smokers with a wide range of smoking histories, suggesting that cigarette smoking did not induce the proliferation of or stimulate the epithelial cells to produce immunoreactive endothelin. Furthermore, cigarette smokers who were known to have hyperresponsive airways had similar proportions of immunoreactive bronchial epithelial cells to those smokers who were known to have non-reactive airways. It is also important to note however that, although one of the patient groups was selected for increased response to methacholine, these patients may not have had clinical asthma. Studies by Moreno *et al*<sup>18</sup> have shown that such a response can be produced when the airways are narrowed secondarily to inflammation and scarring. Our data must not be interpreted as suggesting that endothelin is not important in patients with asthma. Unfortunately we did not have material from a clinically asthmatic non-smoking patient group.

It is also possible that it is not the steady state endothelin expression that is the important feature of bronchial hyperresponsiveness, but the degree to which endothelin production can be stimulated. The presence of endothelin in increased quantities in the bronchoalveolar lavage fluid of a patient during, compared with after, an asthmatic attack would be in keeping with this explanation. In the present study we used an immunostaining technique and therefore have expressed our data quantitatively as the proportion of immunoreactive epithelial cells. It is, however, possible that it is not the number of cells which is important but their ability to secrete increased amounts of endothelin – something which we could not assess. A further possibility relates to the mitogenic properties of endothelin; it is possible that the airway smooth muscle has been altered by numerous episodic bouts of endothelin production, and that it is this secondarily

induced airway change which is responsible for the hyperresponsiveness.

We have found that a significant number of adult human bronchiolar epithelial cells express endothelin immunoreactivity, while Giaid *et al*<sup>3</sup> found only scattered immunoreactive cells. The disparity of these results may be explained by one or more of the following factors. Our study was performed on formalin fixed, paraffin embedded lung sections, but Giaid *et al*<sup>3</sup> did not find any difference between formalin or Zamboni's fixation. We used commercial polyclonal antibodies while Giaid used specific laboratory raised antibodies against endothelin I and big endothelin I; it is possible that the antibodies used by Giaid were much more specific than were those used in our study. Finally, it is possible that the lungs in our study were affected by the presence of lung neoplasms. A further study by Giaid and colleagues<sup>10</sup> did find that airway epithelium adjacent to tumours stained positive, although they did not quantitate this change; our sections were, however, specifically taken away from the tumorous area.

In summary, this study shows that there is a wide range in the proportions of endothelin immunoreactive bronchiolar epithelial cells. The proportion of positive cells is not affected by smoking and does not appear to be increased in patients with known hyperreactive airways. Further studies are necessary to determine the role of endothelin in patients with hyper-responsive airways.

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